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(54) Title: MONOCYTE ADHESION

(57) Abstract: The invention relates to a method of assaying leukocyte binding to solid phase heat shock protein (HSP) which comprises contacting HSP on a solid support with a suspension in a suitable medium of monocytes or a cell line having adhesion properties similar to monocytes and quantitating the number of cells bound to the support. The invention also relates to a method of assaying leukocyte binding via the CD14/TLR signalling complex to a target ligand which comprises contacting the ligand on a solid phase with a suspension in a suitable medium of monocytes or a cell line having adhesion properties similar to monocytes or a cell line transfected with complex components and quantitating the number of cells bound to the solid phase. Agents identified as inhibitors in these assays may be useful in the treatment or prevention of atherosclerosis or related clinical disease and/or for the treatment of conditions where stressed cells in a diseased tissue are targeted by chronic inflammatory responses.



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MONOCYTE ADHESION

Field of Invention

- 5 This invention relates to an assay for the identification of agents that inhibit the adhesion of monocytes to heat shock proteins (HSP) and to agents that inhibit the binding of monocytes to HSP for use in therapy, in particular in the prophylaxis and treatment of atherosclerosis, related clinical disorders and other conditions.

10 Background to the Invention

Atherosclerosis is the underlying disease process responsible for vascular conditions that causes the death of over one third of the population of the Western world. The adhesion of blood monocytes to the wall of susceptible arteries, and their
15 subsequent migration into the wall, are now accepted stages in the pathogenesis of atherosclerosis. Analogy with inflammation and the identity of the distribution of this cellular traffic with the focal occurrence of lesions in the arterial tree are evidence that these monocyte events are critical regulatory stages in the development of the disease. In both atherosclerosis and inflammation, there is focal increased expression of
20 endothelial adhesion molecules in the affected areas. Multiple molecules have been identified as active in inducing monocyte adhesion, and initially similar molecules such as endothelial ICAM-1 binding to monocyte β_2 integrins were implicated in both conditions (1).

25 Beekhuisen and van Furth (2) were the first to identify a novel monocyte molecule, CD14, as involved in monocyte adhesion to cultivated endothelium. Soon afterwards Poston and Johnson-Tidey (3) found evidence of a major role for it in the adhesion of monocytes to atherosclerotic arterial wall *ex vivo*. This was done by use of a novel adhesion assay involving monocytes in suspension and histological sections of
30 atherosclerotic plaques. In this assay, there was inhibition of adhesion of monocytes or U937 cells (a monocyte cell line) by a CD14 antibody to as little as 17% of the control. This action of the CD14 antibody provided evidence for a role of CD14 in the adhesion process. The adhesion assay provides a means for identifying therapeutic agents for the

treatment of atherosclerosis, and CD14 inhibitors as agents for the therapy of atherosclerosis (19-21).

Recent discoveries in the fields of microbiology and innate immunity have allowed
5 new perspectives on the likely role of CD14 in adhesion. Although it has been known
for some time that lipopolysaccharide (LPS, bacterial endotoxin) is a ligand for CD14,
and can thereby stimulate monocytes, it was not understood how trans-membrane
signalling was elicited, as CD14 is a protein bound into the membrane by
glycerophosphatidylinositol anchor. Recently, a series of proteins, the Toll like
10 receptors (TLR), related to the *Drosophila* developmental receptor, Toll, have been
identified, which associate with CD14 and mediate its signalling (4). In the human and
the mouse, TLR4 will permit response to LPS (5), and TLR2 induces response to
related bacterial products (6). Further proteins can associate with CD14 and TLR4,
particularly membrane - bound β_2 integrins, to form a large functional signalling
15 complex. Certain strains of mice, C3H/HeJ and C57BL/10ScCr, are hyporesponsive to
LPS, and have been found to have mutations of the TLR4 gene (7). These mice are
resistant to the development of atherosclerosis if modified to become hyperlipidaemic.
TLR4 knockouts also have been created that are unresponsive to LPS (8). The cellular
activation that follows LPS-LBP-CD14 (LBP - LPS binding protein) interactions is
20 known to activate monocyte integrins. The signalling pathway has been found to
involve the G protein Rho, PI 3-kinase and cytohesin -1 (9).

A further important recent discovery is that human heat shock proteins are endogenous
ligands for the CD14/TRL-4 receptor complex. HSP60 is expressed on the surface of
25 activated leukocytes (10) and on endothelial cells, where it is induced by shear stress or
heat, but not by cytokines or oxidised LDL (11, 12, 13). Much is present on the
endothelial cells over human atherosclerotic plaques (14, and own unpublished data).
Both HSP60 and HSP70 will activate human monocytes (15, 16), and the pathways
have been demonstrated to be CD14 and TRL-4- dependent (16, 17). Furthermore
30 Wick's group have observed that co-expression of HSP60 and ICAM-1 was found in
the endothelial cells of LPS stimulated rat aortic organ cultures, and that this correlated
with the adhesion of monocytes and lymphocytes to them (22). Antibodies to several

adhesion molecules were able to block the adhesion, but an HSP60 antibody was without effect.

5 It can be concluded that there is good published evidence that HSP60/70 are capable of activating human monocytes via CD14/ TRL-4. Although it is possible that this interaction may be involved in the pathogenesis of atherosclerosis, its precise role is not known.

Summary of the Invention

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According to one aspect, the present invention provides a method of assaying leukocyte binding to solid phase HSP which comprises contacting HSP on a solid support with a suspension in a suitable medium of monocytes or a cell line having adhesion properties similar to monocytes and quantitating the number of cells bound to the support.

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According to another aspect, the present invention provides a method of identifying inhibitors of the binding of monocytes to HSP which comprises carrying out the assay defined above in the presence and in the absence of a potential inhibitor and assessing the effect of the potential inhibitor on the extent of monocyte binding. For example the
20 assay may be carried out with or without preincubating the monocytes and/or the HSP with the potential inhibitor.

25

According to a further aspect, the present invention provides an agent which is characterised by the property of inhibiting binding of monocytes to HSP, e.g. as determined by the assay defined above, for use in therapy. The agent may be used for the treatment or prevention of atherosclerosis or related clinical disease, generally by a method which involves inhibition of the binding of monocytes to arterial tissue. The agent may also be used for the treatment of conditions where stressed cells in a diseased tissue are targeted by chronic inflammatory responses, generally by a method
30 which involves inhibition of the binding of monocytes to the said diseased tissue.

The present invention also provides a method for the treatment or prevention of atherosclerosis or a related clinical disease, generally by a method which involves

inhibition of the binding of monocytes to arterial tissue, which comprises administering to a patient in need thereof an effective amount of an agent which is characterised by the property of inhibiting binding of monocytes to HSP.

- 5 The present invention further provides a method for the treatment of a condition in which stressed cells in a diseased tissue are targeted by a chronic inflammatory response, generally by a method which involves inhibition of the binding of monocytes to the said diseased tissue, which comprises administering to a patient in need thereof an effective amount of an agent which is characterised by the property of inhibiting
10 binding of monocytes to HSP.

The present invention also provides the use of an agent which is characterised by the property of inhibiting binding of monocytes to HSP for the manufacture of a medicament for use in the treatment or prevention of atherosclerosis or related clinical
15 disease, generally by a method which involves inhibition of the binding of monocytes to arterial tissue.

The present invention further provides the use of an agent which is characterised by the property of inhibiting binding to HSP for the manufacture of a medicament for use in
20 the treatment of a condition in which stressed cells in a diseased tissue are targeted by a chronic inflammatory response, generally by a method which involves inhibition of the binding of monocytes to the said diseased tissue.

The present invention also provides a method for the identification of an adhesion
25 ligand of the CD14/TLR complex which comprises contacting with the ligand on a solid phase a suspension of monocytes or a cell line with having adhesion properties similar to monocytes, with and without the addition of antibodies against components of the CD14/TLR complex.

30 The present invention further provides a method for the identification of an adhesion ligand of the CD14/TLR complex which comprises contacting with the ligand on a solid phase a suspension of transfected cells expressing the complex, and comparing adhesion with untransfected cells.

According to a further aspect, the present invention provides a method of assaying leukocyte binding via the CD14/TLR signalling complex to a target ligand which comprises contacting the ligand on a solid phase with a suspension in a suitable
5 medium of monocytes or a cell line having adhesion properties similar to monocytes, or a cell line transfected with the complex components, and quantitating the number of cells bound to the solid phase ligand.

According to another aspect, the present invention provides a method of identifying
10 inhibitors of the binding of monocytes or other CD14/TLR complex bearing cells to a target ligand via the CD14/TLR signalling complex which comprises carrying out the assay defined above in the presence and in the absence of a potential inhibitor and assessing the effect of the potential inhibitor on the extent of cell binding to the ligand. For example the assay may be carried out with or without preincubating the cells and/or
15 the target ligand with the potential inhibitor.

According to a further aspect, the present invention provides an agent which is characterised by the property of inhibiting binding of monocytes via the CD14/TLR signalling complex to CD14/TLR ligands, e.g. as determined by the assay defined
20 above, for use in therapy. The agent may be used for the treatment or prevention of atherosclerosis or related clinical disease, generally by a method which involves inhibition of the binding of monocytes to arterial tissue. The agent may also be used for the treatment of conditions where stressed cells in a diseased tissue are targeted by chronic inflammatory responses, generally by a method which involves inhibition of
25 the binding of monocytes to the said diseased tissue.

The present invention also provides a method for the treatment or prevention of atherosclerosis or a related clinical disease, generally by a method which involves inhibition of the binding of monocytes to arterial tissue, which comprises administering
30 to a patient in need thereof an effective amount of an agent which is characterised by the property of inhibiting binding of monocytes via the CD14/TLR signalling complex to CD14/TLR ligands.

The present invention further provides a method for the treatment of a condition in which stressed cells in a diseased tissue are targeted by a chronic inflammatory response, generally by a method which involves inhibition of the binding of monocytes to the said diseased tissue, which comprises administering to a patient in need thereof
5 an effective amount of an agent which is characterised by the property of inhibiting binding of monocytes via the CD14/TLR signalling complex to CD14/TLR ligands.

The present invention also provides the use of an agent which is characterised by the property of inhibiting binding of monocytes via the CD14/TLR signalling complex to
10 CD14/TLR ligands for the manufacture of a medicament for use in the treatment or prevention of atherosclerosis or related clinical disease, generally by a method which involves inhibition of the binding of monocytes to arterial tissue.

The present invention further provides the use of an agent which is characterised by the
15 property of inhibiting binding via the CD14/TLR signalling complex to CD14/TLR ligands for the manufacture of a medicament for use in the treatment of a condition in which stressed cells in a diseased tissue are targeted by a chronic inflammatory response, generally by a method which involves inhibition of the binding of monocytes to the said diseased tissue.

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DETAILED DESCRIPTION OF THE INVENTION

One hypothesis is that the activation of human monocytes by HSP via CD14/ TRL-4 would permit enhanced adhesion of endothelial ICAM-1 to monocyte β_2 and β_3
25 integrins by activation of those molecules, and hence enhance the adhesion of monocytes to the arterial wall. To establish exactly the role of CD14 and HSPs in allowing the adhesion of monocytes to the arterial wall, two assays were employed:

1) investigation of the role of HSP in the adhesion of monocytes to histological sections
30 of human atherosclerosis. This assay can be conducted as described previously (3).

2) a novel *in vitro* assay was devised to investigate the adhesion molecules involved in the adhesion of monocytes to solid phase proteins, including vitronectin (a β_3 integrin ligand) and HSP60.

- 5 The present invention is applicable generally to HSPs but will be described particularly with reference to HSP60. A similar adhesion interaction may exist with HSP70 and with other HSPs.

Investigation of the binding of the U937 monocyte cell line cells to sections of human
10 atherosclerotic plaque showed that it could be inhibited by four different monoclonal antibodies to HSP60, strongly suggesting that this molecule has a role in the adhesion process. It can be deduced that HSP60 is likely to have a major role in the adhesion of monocytes to atherosclerotic plaques *in vivo*. Although there has been extensive
15 previous research on the involvement of HSP60 in the pathogenesis of atherosclerosis, for example in the investigation of immune responses to it by Wick's group, it has not been previously suggested that it is an adhesion molecule. Likewise its interaction with CD14 has been previously demonstrated by others to give rise to activation of monocytes, but no evidence was obtained that this gave rise to an adhesion interaction. It is however known that soluble bacterial lipopolysaccharide will induce monocyte
20 adhesiveness to solid phase ICAM-1 by signalling through CD14 (9).

The assay for the binding of monocytes to HSP may be carried out using solid HSP, for example recombinant human HSP60, coated onto a solid substrate, for example the wells of a conventional well plate. A suitable monocyte cell line is activated and
25 labelled, for example with fluorescein and incubated with the HSP on the solid support. The extent of binding of the monocytes to the HSP can be determined by means of the label, for example by fluorescence spectrophotometry. According to one embodiment of the invention, the effect of potential inhibitors of the binding of monocytes to HSP can be determined and inhibitors can be identified by carrying out the assay in the
30 presence and absence of a potential inhibitor. The inhibitor can be added during the incubation of the monocytes with the HSP or the monocytes and/or the HSP can be pre-incubated with the potential inhibitor.

The involvement of the CD14/TLR signalling complex, with or without involvement of HSP, in the binding of monocytes to the atherosclerotic plaque and other diseased tissue where stressed cells are targeted by a chronic inflammatory response, also provides the potential for novel approaches to the therapy of such conditions.

5

Monocyte bonding to solid phase ligands of the CD14/TLR signalling complex can be assayed by the same general method as described herein for assaying the binding of monocytes to HSP. In instances where the specificity of the ligand for the CD14/TLR complex is uncertain, antibodies to complex components e.g. to TLR-4 can be added to the assay, when inhibition of binding by the antibody will indicate involvement of the specific component. Alternatively, cell lines not bearing complex components can be transfected with the components, or with empty vector, and the adhesive ability compared. For example, K562 cells can be transfected with CD14.

15 Carrying out the assay in the presence and absence of potential inhibitors of the binding of monocytes via the CD14/TLR signalling complex also enables such inhibitors to be identified which agents may then have potential application as therapeutic agents. The diseases for which such agents could provide a therapeutic approach are the same as those indicated herein for inhibitors of the binding of monocytes to HSP.

20

Any monocyte-like cell line can be used in the method according to the invention. A monocyte-like cell line is a cell line which has adhesion properties similar to human monocytes so that it adheres to HSP (for example solid phase recombinant HSP60) in a similar manner to human monocytes. It may be produced by transfection of other cell types with CD14/TLR complex components. The adhesion properties of monocytes are, in turn, determined by the adhesion receptors on the surface of the cell.

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The monocyte-like cell line is preferably a monoclonal cell line. One particularly preferred monocyte-like cell line is the U937 histiocytic lymphoma cell line (27) available to the public from ATCC number CRL 1593. The U937 cell line was first described by Sunderstrom & Nilsson (28). An alternative monocyte-like cell line is the THP-1 monocyte cell line (29) available to the public from ATCC number TIB 202.

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The above monoclonal monocyte-like cell lines can be grown by standard methods in cell culture medium such as RPMI medium and will generally be used according to the invention in suspension in that or a similar medium. In the case of the U937 cell line, a preferred cell culture medium is RPMI medium containing 10% fetal calf serum and
5 this medium can also be used for the assay. The monoclonal monocyte-like cells are preferably activated in order to increase adhesion, for example by use of a phorbol ester. According to one embodiment of the invention U937 cells can be activated by use of phorbol myristyl acetate (PMA), for example by suspension in tissue culture medium containing 10ng/ml phorbol myristyl acetate for 16-24 hours at 37°C.

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Whilst monoclonal cell lines are preferred, normal human monocytes can also be used in the application of the method according to the invention. Normal human monocytes can be prepared from heparinised human blood by centrifuging on a Ficoll-Hypaque density gradient to isolate mononuclear leukocytes, followed by adhesion to plastic
15 tissue culture flasks. Alternatively, monocytes can be isolated by negative selection using antibody coated beads. Furthermore, by use of monocytes derived from patients' blood, the assay can also be employed to assess the adhesive properties of monocytes in patients with atherosclerotic or other disease.

20 The assay according to the present invention is valuable for a number of purposes. The involvement of adhesion molecules in the entry of monocytes into atherosclerotic foci may be of profound significance as it appears to be a vital mechanism in this initial event in the generation of the disease. There is reason to suppose that once monocyte entry has started, it may be self-perpetuating, as factors produced by the monocyte-
25 derived macrophages may elicit further formation of endothelial adhesion molecules. As well as providing a means of investigating the mechanism of monocyte entry, the method according to the invention has important uses in the development of therapeutic approaches to the treatment of atherosclerosis and other conditions.

30 Agents that can inhibit the process of adhesion of monocytes to HSP, in particular HSP60 and HSP70, are candidates for use as therapeutic agents against human atherosclerosis and other conditions. The method according to the invention can be used for screening possible inhibitory agents with the potential for the development of

therapeutic approaches, for example against human atherosclerosis. Assay for adhesion of monocyte-like cells in the presence and the absence of a potential inhibitory agent will identify those agents which inhibit the adhesion process. Alternatively the cell suspension and/or the HSP can be preincubated with a potential inhibitory agent.

5

Involvement of CD14 in monocyte adhesion in atherosclerosis has been the subject of previous patents (19, 20, 21). However the new evidence of an adhesion interaction with HSP60 *in vitro* explains the nature of the adhesion, and provides an assay suitable for high throughput screening for the identification of antagonists. Such antagonists could be lead compounds for the development of therapeutic agents for treating diseases where this adhesion interaction may be important, for instance in atherosclerosis.

10

CD14 is involved in responses to bacterial endotoxin, but evidence from mouse experiments unexpectedly indicates that it may not be of significance in protection against infection (23), or even be deleterious, as anti-CD14 antibody treatment gave therapeutic benefit in a rabbit/endotoxin model (24). Blocking of CD14 signalling might therefore be valuable in treating human sepsis and related conditions such as adult respiratory distress syndrome and toxic shock syndrome.

15

20

The CD14/ HSP60 interaction may have wide involvement in conditions where stressed cells are targeted by chronic inflammatory responses e.g. burns, rheumatoid arthritis, diabetes, Alzheimer's disease, multiple sclerosis, inflammatory bowel disease and in tissues affected by ischaemia. Genetic analysis suggests that CD14 is implicated in cirrhosis of the liver (25) and atopy (26), thus liver disease, e.g. alcoholic liver disease, allergic rhinitis and asthma are further potential fields of use.

25

In some cases, it may be possible to administer an agent which is characterised by the property of inhibiting binding of monocytes to HSP to a patient as the raw substance but the agent will generally be presented as a pharmaceutical composition. In this context a pharmaceutical composition comprises at least one agent which inhibits binding of monocytes to HSP (referred to herein as the "active ingredient") with one or more pharmaceutically acceptable carriers or diluents. The carrier(s) or diluents(s)

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must be "acceptable" in the sense of not having any deleterious effect on the patient and being compatible with other components of the formulation. The pharmaceutical composition may also contain other therapeutic ingredients having the same or a different therapeutic effect from the active ingredient, for example agents having an effect on the heart or circulation, such as anti-coagulants or anti-hypertensives.

In the case of small chemical molecules, the active ingredient may be formulated for administration by any suitable means provided that it is delivered to the circulation in a manner such that monocyte/HSP adhesion in the vicinity of atherosclerotic plaque or at potential sites of atherosclerotic plaque or at the site of other diseased tissue being treated can be inhibited. Examples of suitable forms of administration include oral, parenteral, rectal or intranasal, e.g. inhalation.

A pharmaceutical composition for oral administration may take the form of, for example, tablets or capsules and may be prepared by processing the active ingredient in a conventional manner together with one or more pharmaceutically acceptable excipients. Tablets may be prepared by compression or moulding in known manner and suitable excipients include binding agents, fillers, lubricants, disintegrants and wetting agents. Tablets and capsules may be coated in known manner, for example to provide slow or controlled release of the active ingredient.

Liquid preparations for oral administration may take the form, for example, of solutions, syrups or suspensions or may be presented as a dry product for reconstitution with water or another suitable vehicle prior to use.

Compositions for parenteral administration include aqueous and non-aqueous sterile injection solutions which may be formulated in known manner. The formulations may be presented in unit-dose or multi-dose containers, for example, ampoules or vials, or may be stored in a lyophilised condition suitable for reconstitution by addition of sterile liquid, for example water for injection.

Compositions for rectal administration may be presented in forms such as suppositories or retention enemas which may be formulated in known manner.

Compositions for intranasal administration may be formulated for administration via a metered dose or unit device or as a powder including a suitable carrier for administration using an appropriate delivery system.

5

Active ingredients which are larger molecules such as proteins, for example antibodies, will generally also be administered to patients in the form of a pharmaceutical composition which preferably includes, in addition to the active ingredient, a physiologically acceptable carrier or diluent, possibly in admixture with one or more other agents such as other drugs, including antibiotics or agents which have an effect on the heart or circulation. Suitable carriers include physiological saline and phosphate buffered saline. Alternatively, the active ingredient may be lyophilised and reconstituted before use by the addition of an aqueous buffered solution. Routes of administration of the active ingredient include intravenous, intramuscular, subcutaneous and intraperitoneal injection or delivery.

The method by which the active ingredient is used in the treatment or prevention of atherosclerosis or other conditions will depend on the nature of the agent. Small chemical molecules may be used prophylactically over long periods by subjects at risk of atherosclerosis or other conditions which can be treated according to the invention. Proteins, such as antibodies, carry more risk of an adverse reaction from the subject's immune system and are more suitable for short term therapy of patients at particular risk in special circumstances, for example following heart transplantation. In all cases the precise dose will be at the discretion of the attendant physician but will depend on the nature of the agent and a number of other factors including the age and sex of the patient, the condition of the patient and the severity of the disorder being treated.

EXAMPLES

The invention is illustrated further by the following experimental work.

Adhesion of monocytes to the arterial endothelium is a major mechanism in the initiation and development of atherosclerotic plaques. It is possible to investigate the

mechanisms of this adhesion *in vitro* by measuring the binding of the U937 monocyte cell line after PMA activation on to unfixed histological frozen transverse sections of human atherosclerotic lesions (Poston & Johnson-Tidey, 1996). The PMA activated U937 cell has proved to be a good model of the blood monocyte in all work to date, with no differences in behaviour detected.

Carotid endarterectomy specimens were obtained with the permission of the Ethical Committees of St Thomas' and Guy's Hospitals. The adhesion assay was performed as previously described (3, 19-21). Briefly, a suspension of PMA activated (10ng/ml, 16 hours) U937 cells were added at 10^7 /ml to 8 micron cryostat sections of the arteries and incubated at 37°C for 40 minutes with agitation on a rotating table at 60rpm. The sections were then washed by 6 quick dips into PBS, and the adherent cells fixed with 4% paraformaldehyde for 15 minutes. The endothelium was then stained by direct immunohistochemistry for von-Willebrand factor using a peroxidase conjugated antibody. Binding was quantitated over intact endothelium as cells/length.

Two monoclonal antibodies to epitopes of HSP60 expressed on cell surface membranes (ML-30 and II-13, 20 µg/ml) significantly reduced ($p < 0.05$, ANOVA) U937 cell adhesion to the arterial endothelium to 37 ± 16 (SD) and $36 \pm 8\%$ of control (4 patients, each artery measured 4 times). Two further antibodies (LK-1 and 4B9/89) to other epitopes similarly significantly reduced adhesion to 59 ± 22 and $57 \pm 30\%$ of control. Adhesion to the intimal connective tissue was also inhibited.

Binding of fluorescein labelled U937 cells to solid phase recombinant HSP60 was investigated to test the idea that the adhesion interaction is between plaque HSP60 and monocyte CD14. Black 96 well plates (Corning) were coated with recombinant human HSP60 (Sigma) at 3.2 - 10 µg/ml, or other proteins, by incubation for 16 hours at 4°C in bicarbonate buffer pH 9.0. The protein solution was then aspirated, and the plates washed three times by immersion in a bath of PBS, and gently flicking out the contents after each wash. U937 cells were activated for 16 hours with PMA 10ng/ml at 37°C in RPMI with 10% fetal calf serum, and then washed in the same medium. The U937 cells were recentrifuged and the pellet added to the same medium containing 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester 0.1mM (C-1157, Molecular Probes),

and incubated for 30 minutes at 37°C in the dark. This fluorescein compound is a highly convenient cell label, as it is only taken up into viable cells, where it persists. The cells were then washed three times in medium, and added at a concentration of $1-3.2 \times 10^6/\text{ml}$ in $100\mu\text{L}$ with or without inhibitors, to the black 96 well plates. The plates
5 were then incubated for 1 hour at 37°C in the dark. After incubation, the plate was aspirated, and washed three times by immersion in a PBS bath, and gently flicking out the contents. Cell binding was then measured in an automatic fluorescence spectrophotometer, and expressed as percentage of total cells bound by reference to control wells containing the original cell suspension. Experiments were performed in
10 triplicate.

$37 \pm 7\%$ of the total U937 cells bound to HSP60 (3 experiments, triplicate measurements), which was comparable with that induced by fibronectin (30%) or vitronectin (42%). Binding to control uncoated wells was $<10\%$. The HSP60
15 interaction was significantly inhibited ($p < 0.05$, t test) by CD14 antibody, to $6.3 \pm 5.5\%$ of cells at $1.6\mu\text{g}/\text{ml}$. In contrast the same antibody had little effect on binding induced by fibronectin ($97 \pm 20\%$ of the controls) or that of vitronectin ($100\% \pm 8\%$). Furthermore mixed coating of plates with vitronectin and varying amounts of HSP60 gave no evidence of HSP60 enhancing adhesion over either agent alone.

20

In conclusion, HSP60 behaves as a monocyte binding adhesion molecule through its affinity for CD14 and/or the associated receptor complex, and this interaction can be implicated in the adhesion of monocytes to human atherosclerotic plaques. No evidence was obtained of any synergistic action of HSP60 with vitronectin in promoting
25 adhesion, suggesting that under the conditions employed any CD14 dependent activation of monocyte vitronectin-binding integrins, such as of $\alpha\text{-v}\beta\text{-3}$ integrin, was unable to influence adhesion. The involvement of HSP60 in the adhesion process contrasts with the previous report by Seitz et al. (22).

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CLAIMS:

1. A method of assaying leukocyte binding to solid phase heat shock protein (HSP) which comprises contacting HSP on a solid support with a suspension in a
5 suitable medium of monocytes or a cell line having adhesion properties similar to monocytes and quantitating the number of cells bound to the support.
2. A method according to claim 1 wherein the HSP is HSP60.
- 10 3. A method according to claim 1 or 2 wherein the cell line having adhesion properties similar to monocytes is a monoclonal cell line.
4. A method according to claim 3 wherein the cell line is U937.
- 15 5. A method of identifying inhibitors of the binding of monocytes to HSP which comprises carrying out the assay claimed in any of claims 1 to 4 in the presence and in the absence of a potential inhibitor and assessing the effect of the potential inhibitor on the extent of monocyte binding.
- 20 6. A method according to claim 5 wherein the assay is carried out with or without preincubating the monocytes and/or the HSP with the potential inhibitor.
7. An agent which is characterised by the property of inhibiting binding of monocytes to HSP for use in therapy.
- 25 8. An agent as claimed in claim 7 for the treatment or prevention of atherosclerosis or related clinical disease by a method which involves inhibition of the binding of monocytes to arterial tissue.
- 30 9. An agent as claimed in claim 7 for the treatment of conditions where stressed cells in a diseased tissue are targeted by chronic inflammatory responses by a method which involves inhibition of the binding of monocytes to the said diseased tissue.

10. An agent as claimed in claim 9 for the treatment of human sepsis, adult respiratory distress syndrome, toxic shock syndrome, burns, rheumatoid arthritis, diabetes, Alzheimer's disease, multiple sclerosis, inflammatory bowel disease, ischaemia, liver disease, allergic rhinitis and asthma.

5

11. A method for the treatment or prevention of atherosclerosis or a related clinical disease by a method which involves inhibition of the binding of monocytes to arterial tissue, which comprises administering to a patient in need thereof an effective amount of an agent which is characterised by the property of inhibiting binding of monocytes to HSP.

10

12. A method for the treatment of a condition in which stressed cells in a diseased tissue are targeted by a chronic inflammatory response by a method which involves inhibition of the binding of monocytes to the said diseased tissue, which comprises administering to a patient in need thereof an effective amount of an agent which is characterised by the property of inhibiting binding of monocytes to HSP.

15

13. A method according to claim 12 for the treatment of human sepsis, adult respiratory distress syndrome, toxic shock syndrome, burns, rheumatoid arthritis, diabetes, Alzheimer's disease, multiple sclerosis, inflammatory bowel disease, ischaemia, liver disease, allergic rhinitis and asthma

20

14. Use of an agent which is characterised by the property of inhibiting binding of monocytes to HSP for the manufacture of a medicament for use in the treatment or prevention of atherosclerosis or related clinical disease by a method which involves inhibition of the binding of monocytes to arterial tissue.

25

15. Use of an agent which is characterised by the property of inhibiting binding to HSP for the manufacture of a medicament for use in the treatment of a condition in which stressed cells in a diseased tissue are targeted by a chronic inflammatory response by a method which involves inhibition of the binding of monocytes to the said diseased tissue.

30

16. Use according to claim 15 for the manufacture of a medicament for the treatment of human sepsis, adult respiratory distress syndrome, toxic shock syndrome, burns, rheumatoid arthritis, diabetes, Alzheimer's disease, multiple sclerosis, inflammatory bowel disease, ischaemia, liver disease, allergic rhinitis and asthma.

5

17. A method for the identification of an adhesion ligand of the CD14/TLR complex which comprises contacting with the ligand on a solid phase a suspension of monocytes or a cell line with having adhesion properties similar to monocytes, with and without the addition of antibodies against components of the CD14/TLR complex.

10

18. A method for the identification of an adhesion ligand of the CD14/TLR complex which comprises contacting with the ligand on a solid phase a suspension of transfected cells expressing the complex, and comparing adhesion with untransfected cells.

15

19. A method of assaying leukocyte binding via the CD14/TLR signalling complex to a target ligand which comprises contacting the ligand on a solid phase with a suspension in a suitable medium of monocytes or a cell line having adhesion properties similar to monocytes or a cell line transfected with complex components and quantitating the number of cells bound to the solid phase.

20

20. A method according to claim 19 wherein the cell line having adhesion properties similar to monocytes is a monoclonal cell line.

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21. A method according to claim 20 wherein the cell line is U937.

22. A method according to claim 20 wherein the cell line is a transfected cell.

23. A method of identifying inhibitors of the binding of monocytes or other CD14/TLR complex bearing cells to a target ligand via the CD14/TLR signalling complex which comprises carrying out the assay claimed in any of claims 19 to 22 in the presence and in the absence of a potential inhibitor and assessing the effect of the potential inhibitor on the extent of cell binding to the ligand.

30

24. A method according to claim 23 wherein the assay is carried out with or without preincubating the monocytes and/or the target ligand with the potential inhibitor.

5 25. An agent which is characterised by the property of inhibiting binding of monocytes via the CD14/TLR signalling complex to CD14/TLR ligands for use in therapy.

26. An agent as claimed in claim 25 for the treatment or prevention of
10 atherosclerosis or related clinical disease by a method which involves inhibition of the binding of monocytes to arterial tissue.

27. An agent as claimed in claim 25 for the treatment of conditions where stressed cells in a diseased tissue are targeted by chronic inflammatory responses by a method
15 which involves inhibition of the binding of monocytes to the said diseased tissue.

28. An agent as claimed in claim 27 for the treatment of human sepsis, adult respiratory distress syndrome, toxic shock syndrome, burns, rheumatoid arthritis, diabetes, Alzheimer's disease, multiple sclerosis, inflammatory bowel disease,
20 ischaemia, liver disease, allergic rhinitis and asthma.

29. A method for the treatment or prevention of atherosclerosis or a related clinical disease by a method which involves inhibition of the binding of monocytes to arterial tissue, which comprises administering to a patient in need thereof an effective amount
25 of an agent which is characterised by the property of inhibiting binding of monocytes via the CD14/TLR signalling complex to CD14/TLR ligands.

30. A method for the treatment of a condition in which stressed cells in a diseased tissue are targeted by a chronic inflammatory response by a method which involves
30 inhibition of the binding of monocytes to the said diseased tissue, which comprises administering to a patient in need thereof an effective amount of an agent which is characterised by the property of inhibiting binding of monocytes via the CD14/TLR signalling complex to CD14/TLR ligands.

31. A method according to claim 30 for the treatment of human sepsis, adult
respiratory distress syndrome, toxic shock syndrome, burns, rheumatoid arthritis,
diabetes, Alzheimer's disease, multiple sclerosis, inflammatory bowel disease,
5 ischaemia, liver disease, allergic rhinitis and asthma.

32. Use of an agent which is characterised by the property of inhibiting binding of
monocytes via the CD14/TLR signalling complex to CD14/TLR ligands for the
manufacture of a medicament for use in the treatment or prevention of atherosclerosis
10 or related clinical disease by a method which involves inhibition of the binding of
monocytes to arterial tissue.

33. Use of an agent which is characterised by the property of inhibiting binding of
monocytes via the CD14/TLR signalling complex to CD14/TLR ligands for the
15 manufacture of a medicament for use in the treatment of a condition in which stressed
cells in a diseased tissue are targeted by a chronic inflammatory response by a method
which involves inhibition of the binding of monocytes to the said diseased tissue.

34. Use according to claim 33 for the manufacture of a medicament for the
20 treatment of human sepsis, adult respiratory distress syndrome, toxic shock syndrome,
burns, rheumatoid arthritis, diabetes, Alzheimer's disease, multiple sclerosis,
inflammatory bowel disease, ischaemia, liver disease, allergic rhinitis and asthma.